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(54) Title: POLYMER COMPOSITION OF PROTEINS, POLYSACCHARIDES AND / OR GLYCERIDES

(57) Abstract: Polymer compositions comprising at least 2 building blocks covalently linked via phenolic residues containing a methoxygroup positioned ortho with respect to the hydroxyl group, wherein the building blocks are selected from protein (P), glycerides (G) and polysaccharides (S) and wherein the covalent linkage via phenolic residues is between P-P, S-S, P-S, P-G, G-G, G-S or combinations thereof, characterised in that at least one of the phenolic residues is covalently bonded to a building block via a Schiff's base, show good emulsifying, thickening, encapsulation and stabilising properties. Hence they are suitable for use in food products, especially emulsions and foams.

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POLYMER COMPOSITION OF PROTEINS, POLYSACCHARIDES AND/OR GLYCERIDES

The current invention relates to a polymer composition comprising at least 2 building blocks covalently linked via phenolic residues containing an ortho methoxygroup.

Background of the invention

Biopolymers are extensively used in food products as emulsifiers, gelling agent, structuring agents, stabilisers, encapsulation agents, thickeners and the like. Especially biopolymers such as starches, protein and triglycerides are well known and are used for example in sauces, margarine, dressings, soups and many other food compositions. Many of the polymers are naturally occurring and show good functionality in relation to the above mentioned features.

However there is still a need for alternative compositions with improved functionality and controlled characteristics. This desire is exemplified in WO-A-96/03440, which discloses a method for gelling or increase of viscosity of aqueous media containing gellable polymeric materials having substituents with phenolic hydroxyl groups. This document specifically discloses the gelation of arabinoxylans or pectins by adding an effective amount of a laccase to an aqueous medium containing these substituents. Furthermore it is disclosed that proteins having one or more tyrosine residues in the amino acid sequence can be gelled by use of a laccase.

The compositions disclosed in WO-A-96/03440 rely on the gelation of single compositions and hence lead to gels with a limited variety in composition and functionality.

EP 1169922 discloses polymers of protein linked to arabinoxylans via oxidative crosslinking of the tyrosine residues and ferulic acid.

US6232101 discloses gellable polymeric materials having substituents with phenolic groups. Crosslinking is between two phenolic groups. It is disclosed that the groups of phenolic polymers are peptides, proteins, e.g. those that include tyrosine amino acid. Furthermore according to this document, polysaccharides and other polymers can be derivatized via ferulic acid esterase. The formation of the covalent ester bonds disclosed herein is however a difficult process which requires presence of enzymes or severe processing conditions if chemical (inter) esterification is used.

US-A-5,374,441 discloses a heat stable fat substitute composition obtained by the oxidative coupling reaction of phenolic acid groups attached to protein chains. The resulting polymers are linked via the naturally present phenolic residues in a protein. The reaction between such residues is relatively slow and entirely dependent on the frequency of tyrosine in the protein.

It is an object of the current invention to provide polymers which can be used in a variety of food products and/or pharmaceutical products and which enlarge the group of currently available polymers for use in foods/pharma. The invention especially relates to polymers suitable for stabilising oil and water containing emulsions and foams and to polymers suitable for encapsulation.

Summary of the invention

It has surprisingly been found that specific polymers of protein, polysaccharides and/or glycerides meet the above objective.

Therefore the invention relates to a polymer composition comprising at least 2 building blocks covalently linked via phenolic residues containing a methoxygroup positioned ortho with respect to the hydroxyl group, wherein the building blocks are selected from protein (P), glycerides (G) and polysaccharides (S) and wherein the covalent linkage via phenolic residues is between P-P, S-S, P-S, P-G, G-G, G-S or combinations thereof, wherein at least one of the phenolic residues is covalently bonded to a building block via a Schiff's base or the reduced form thereof.

The invention further relates to food products containing these polymers and to a method for preparation of these polymers.

In a further aspect the invention relates to use of these compositions for encapsulation and/or targeted delivery of functional molecules.

Detailed description of the invention

The polymers according to the invention are composed of so called building blocks. Building blocks are polymer units, which are covalently linked via two connected phenolic residues. This covalent bond is an essential part of the claimed polymers.

For example WO-A-99/16893 discloses a process wherein phenolic compounds are added to a casein in the presence of an oxidative enzyme. We have found that the resulting compositions do not contain a covalent linkage between phenolic residues. Therefore the compositions according to this publication do not show the properties that are shown by the currently claimed polymers such as the emulsification properties.

The building blocks of the polymers according to the invention are polysaccharides (S), glycerides (G) and proteins (P). Glycerides in the context of the invention are glycerol based molecules wherein the glycerol backbone is covalently linked to at least one residue such as a fatty acid. Well known glycerides are mono, di and tri acyl fatty acid glycerides. Mono- and diglycerides are already known for their emulsifying capacity. Triglycerides are well known texturing agents in products such as margarine, butter, creams.
The glycerides may contain any fatty acid, both naturally occurring and synthetic ones.
Preferred fatty acids have a chain length of from 16 to 18 carbon atoms. Suitable fatty acids include oleic acid, stearic acid, palmitic acid.
Most preferred, the glyceride building block is a diglyceride.

Another building block is protein. Proteins are chains of covalently linked amino acids whereby it is possible to use any desired protein. Preferred proteins contain at least one lysine amino acid residue. Food grade proteins are preferred and hence protein is preferably selected from the group of dairy proteins such as casein and whey protein, vegetable proteins, especially soy protein, and egg protein or gelatin.

The third potential building block is polysaccharide. Any polysaccharide can be used in the polymers according to the invention but preferred are those polysaccharides which in their naturally occurring form contain a phenolic residue such as ferulic acid, vannilic acid, coumaric or cinnamic acid, which are generally linked to the backbone via an acid group and a hydroxyl group of a sugar residue. Sugar beet pectin and arabinoxylanes isolated from cereals are examples of polymers that contain ferulic acid residues in their naturally occurring form. These compositions are described in more detail by Lex Oosterveld , Carbohydrate research 300, 179-181, 1997 and thesis Lex Oosterveld, Landbouwuniversiteit Wageningen Netherlands, 16.12.1997, ISBN 90-5485785-4.

The preferred building blocks are proteins or polysaccharides or a combination thereof.
Even more preferred, either all building blocks are saccharides or all building blocks are proteins.

The building blocks in the polymer are linked via two phenolic residues containing an ortho methyxygroup wherein ortho refers to the position of the methoxy group with respect to the hydroxyl group of the phenolic residue.

It is known in the art that phenolic residues such as ferulic acid when oxidised may lead to formation of crosslinked polymers. Examples of covalent bonds between two phenolic residues are described by Oosterveld (thesis Lex Oosterveld 1997) and in US 5,786,470. Preferably the phenolic residue is derived from the group of vanillic acid, ferulic acid, coniferol. The most preferred phenolic residue is vanillin.

For example the bond is such that a covalent bond results between a free position of the phenolic residue on a first polymer and a free position of the phenolic residue of a second polymer. This is shown in figure 1. Figure 1 is one example of a specific covalent bond between the two phenolic residues. Other forms of covalent bonding are also encompassed within the invention.

The building blocks that are part of the polymers according to the invention are functionalised such that they contain at least one phenolic residue before oxidation. For functionalisation of the phenolic residues, use is made of a phenolic compound containing a methoxy group in ortho position with respect to the hydroxyl group of the phenolic residue. The presence of the ortho methoxy group in these polymers is essential because it increases the reaction speed and efficacy of the coupling between two phenolic groups. Naturally occurring proteins comprising tyrosine do not contain such ortho-methoxy groups.
Another advantage of functionalisation of the building blocks is that it provides control over the degree of substitution. By stoechiometric control of the functionalisation reaction, the amount of Schiff base formed may be controlled.

The functionalisation is preferably such that the amount of phenolic residues in the building blocks is more than the naturally present amount of phenolic residues in these building blocks. For example in proteins, the amount of tyrosine functionalised with a phenolic group via a Schiff base is preferably at about 5% of the total amount of amino acids in the protein. For polysaccharides it is preferred that on average about 1 hydroxylgroup per 3 monomers, more preferred

one hydroxyl group per monomer is functionalised with a phenolic group.

In the polymers according to the invention, at least one, preferably both of the phenolic residues that take part in the covalent phenolic-phenolic bond, are covalently bonded to a building block via a Schiff's base or the reduced form thereof. We have found that this form of covalent bonding has as an advantage that it is a strong bond, which may be formed under mild processing conditions. In the polymers according to the invention the Schiff base line is between the backbone and a phenolic group. The thus bonded phenolic group takes part in the covalent linking between build blocks.

A Schiff's base (also called imine) is represented by the following formula:

$R_1-N=C(R_{2,3})_2$

An imine is defined as the condensation product between a carbonyl (aldehyde or ketone) and an amine. Both the carbonyl and the amine may be aliphatic or aromatic molecules that may be substituted by any group. Hence R1, R2 andR3 may be hydrogen or any substituted or unsubstituted aliphatic or aromatic group. It is preferred to have an ortho methoxy phenolic group at either side of the C=N bond. For coupling it to a polysaccharide or a diglyceride the methoxygroup is preferably at the N side of the C=N bond and in case of a protein preferably at the C side of the C=N bond. However for coupling to a protein in a few exceptions it may be at the N side as well.

In the context of the invention, the reduced form of the imine or Schiff's base, viz. the amine is also covered by the invention. Where in this specification the word Schiff base is used, the reduced form is encompassed therein.

A Schiff's base is preferably formed by condensation of a primary amine and an aldehyde or ketone. In the polymer according to the invention the amine may be originating from the phenolic residue or from the building block. An example of the latter embodiment is where the amine is derived from a lysine and the alpha amine group of the N-terminal amino acid residue of a protein. Another example is the embodiment where the amine is derived from the free amino group of chitosan or another polysaccharide.
In the polymer according to the invention the aldehyde or ketone may be originating from the phenolic residue or the building block. An example of the former embodiment is the aldehyde group of vanillin. An example of the latter group is the aldehyde group of a starch polymer, which may be present naturally or formed by oxidation of a hydroxyl group.

A Schiff's base is generally formed by mixing the reaction ingredients (phenolic molecule such as vanillin and polymer such as protein) under basic conditions, preferably around pH 8, in aqueous environment at room temperature. For the formation of the Schiff base it is required that in the process a drying step is included. This drying step is essential to remove water and thereby drive the condensation reaction to completion. The drying may be carried out in any suitable manner such as freeze drying, spray drying, open air-drying.

An example of the formation of a Schiff's base link between a protein and vanillin is described in WO00/00507 which discloses the Schiff's base product of a protein and aromatic O-hydroxyl aldehyde.

One way to obtain a protein-phenolic residue Schiff's base composition is by mixing the phenolic residue such as vanillin in aqueous medium with protein, adjusting the pH above 8, stir at ambient temperature, followed by overnight storage at -20 °C and drying for example by spray drying or lyophilisation. This procedure results in the formation of a Schiff base link between the ε-NH_2 of lysine residues and/or the alpha NH_2 of the N-terminal amino acid of the protein and aldehyde or acidic groups of the phenolic compound such as the aldehyde group of vanillin.

The introduction of a phenolic residue other than via Schiff's base formation is described below for each of the building blocks.

Polysaccharides

Well known esterification conditions can be used for introduction of a phenolic group on the polysaccharide chain.

Protein

Functionalisation of a protein is e.g. described above and in the examples.

Glycerides

Di/mono -glycerides containing at least one phenolic residue are preferably prepared by interesterification of a triglyceride oil with the ester form of the phenolic residue. Examples of the latter are ferulic acid ester with para position esterified to ethanol, vanillic acid ester with para

position esterified to an alcohol or caffeic acid ester with paraposition esterified to an alcohol.
Said interesterification reaction can be carried out with chemical or enzymatic catalysts. The use of lipase as catalyst is preferred.

The polymer according to the invention comprises at least two building blocks. Embodiments wherein n building blocks are linked to obtain a large linear polymer are also encompassed within the invention. Further encompassed in the invention are polymers wherein building blocks are linked via at least 2 phenolic residues or by other (non) covalent linkages. It will be appreciated that the higher the amount of phenolic residues in a starting polymer, the more covalent linkages can be formed between one polymer and another. The polymers according to the invention can be both linear and branched polymers.

In the polymers the covalent linkage is between P-P, S-S, P-G, P-S, G-G, G-S, or combinations thereof.
These alternative embodiments are described below.

Figures 1, 2, 3 show examples of covalent linkages and resulting polymers. In these figures, P is protein, S is a saccharide and R is alkyl group.
In these figures, the alternative embodiments P-P (1), P-G (2a,b) and S-G (3a,b,c) are exemplified. The alternatives of coupling the two polymers via Schiff bases (Schiff) or esters are included: e.g. figure 2a shows protein-Schiff-phenolic group-phenolic group-ester-glyceride, figure 2b shows protein-schiff-base-phenolic group-phenolic group-schiff base-glyceride, figure 3a shows polysaccharide-schiff-phenolic-phenolic-schiff-glyceride, figure 3b shows polysaccharide-

schiff-phenolic-phenolic-ester-glyceride; figure 3c shows polysaccharide-ester-phenolic-phenolic-schiff-glyceride.

The covalent bonds between building blocks in the polymers according to the invention, may serve to link two build blocks together by one or more covalent bonds. The latter form, whereby more than one covalent bond is formed between two individual polymers is believed to lead to crosslinked compositions with beneficial product properties such as strong gel formation, possibility to serve as encapsulates for other compounds, stabilisation of aerated products, prevention of syneresis, water retention in gel type products, and functionality as a water barrier.

In a first embodiment the polymer comprises proteins linked to other proteins via a covalent di-phenolic linkage. These polymers may consist of two or more different proteins linked together or the same protein may be linked in a series. According to an alternative embodiment the proteins that are linked are enzymes showing different functionality. This type of polymers shows a combination of functionalities and is therefore an attractive ingredient. Examples of combinations of proteins are: casein-casein, casein- βlactoglobulin, casein-albumin, albumin-albumin, and glycinin-casein.

In a second embodiment, the polymer comprises covalently linked proteins and glycerides, preferably diglycerides. These polymers when branched form fat-protein networks which may be applied as structuring agents. Another application of these compositions is as encapsulating agents.

In another embodiment the polymer comprises covalently linked polysaccharides and glycerides, preferably diglycerides. These compositions were found to be suitable emulsifiers that may be used to stabilise oil and water containing compositions. In such compositions the polymers are found at the oil/water interface whereby the fatty acid chains are in the oil phase and the polysaccharide part is in the aqueous phase. Preferred glycerides for this purpose are diglycerides containing fatty acids derived from sunflower oil, soybean oil, rapeseed oil, maizgerm oil, olive oil, line oil, peanuts, cottonseed oil, and safflower oil, butter fat.
Preferred polysaccharides for this purpose are selected from the group comprising pectins from sugar beet, arabinoxylans, and starches.

According to another embodiment the polymer comprises protein and polysaccharide building blocks. This polymer is especially suitable for encapsulation of ingredients in a product. In an alternative embodiment these compositions are used for foam stabilisation, emulsification or as thickeners. In these embodiments the preferred protein is milk protein and the preferred polysaccharide is selected from the group of pectins and gums such as locust bean gum, guar gum.

According to yet another embodiment, the polymer comprises polysaccharide building blocks. Such polymers, e.g. when based on chitosan were found to be very suitable encapsulating compositions once crosslinked via multiple covalent bonds between multiple phenolic residues.

As indicated above, the polymers according to the invention may suitably be applied in food products, whereby depending on their composition the properties of the food product can be

influenced. Their use is especially recommended in oil and water containing compositions.
Therefore in a further aspect the invention relates to food products containing said polymers.

The following beneficial characteristics of these compositions have been identified.
$(G-G)_n$ which are referred to as acyl-glycerol-phenolic acid hybrids, have superior emulsifying properties especially in oil water compositions of relatively low fat content (i.e. between 10 and 60 wt% fat). Furthermore these compositions may be used as texturising agents, imparting viscosity and/or structure to a composition.

Furthermore these compositions, especially (S-S)n, (P-G)n, and(P-S)n (n equal to or higher than 1) containing polymers are suitable for encapsulation of ingredients such as flavour compounds or functional ingredients.
The polymer molecules according to the invention may be used to create networks to encapsulate or capture functional molecules like drugs and nutritional compounds. These functional molecules may be hydrophilic or hydrophobic. The encapsulated functional molecules may be released at targeted sites in the gastro intestinal tract. It is believed that the driving force to keep the functional molecules encapsulated or entrapped, is the molecular interaction between the polymer network and the entrapped or encapsulated molecule. Examples of such interaction is vd Waals bonding, hydrogen bonds and electrostatic interactions. Once the polymer based network looses integrity the interactions will reduce or cease to exist and the entrapped or encapsulated molecule may be released.

According to one embodiment, positively charged functional molecules are kept within a protein-containing polymer, based on electrostatic interactions between the positively charged molecule and negatively charged carboxyl groups of the protein. According to another embodiment, negatively charged functional molecules are kept within a saccharide-containing polymer, based on electrostatic interactions between the negatively charged molecule and a positively charged amino group of the saccharide such as chitosan.

According to a further embodiment, more specific interactions for encapsulation may be introduced by covalent coupling of functional groups to the building blocks of the polymer according to the invention. The entrapment of hydrophobic molecules in the network may be enhanced by hydrophobic functionalisation of the building blocks, e.g. via covalently bound diglycerides of fatty acids.

The release of the entrapped or encapsulated molecules may be triggered by the disintegration of the structure of the polymer, which may be induced by enzymatic degradation of (part of) the polymers of the network, by e.g. proteolytic, amylolytic or lipolytic activity in the gastrointestinal tract.

Hence on basis of these polymers encapsulates may be made which are e.g. suitable for pharmaceutical applications. We have found that the resulting polymers are stable in the gastro-intestinal tract and are only broken down with release of the encapsulate, once specific enzymes are present. For this application, especially mixed polymers, which are based on different individual building blocks, are very suitable.

Therefore in a preferred embodiment, the invention relates to use of a polymer according to the invention for targeted delivery of a functional molecule.
According to one embodiment, a functional molecule is encapsulated in a saccharide-protein Schiff base crosslinked polymer according to the invention. The functional molecule may be released under the action of pancreatic enzymes. This is shown in example 1.

In a further aspect the invention relates to an oil in water emulsion comprising a polymer according to invention.

In a preferred embodiment, the invention relates to an oil and water emulsion comprising from 20 to 80 wt% fat and from 0.1 to 10 wt% of said polymer. Even more preferred, the polymer comprises covalently linked glyceride and polysaccharide.

The polymers according to the invention are prepared from their building blocks under suitable circumstances to form a covalent bond. In a further aspect the invention relates to a method for the preparation of said polymer, wherein a composition comprising the building blocks is treated with an oxidative enzyme. This treatment can be carried out in presence or absence of a solvent. For most cases, the use of an aqueous medium is preferred. However for example in case of the preparation of a glyceride-glyceride hybride polymer, no solvent is needed as long as the oxidative enzyme is functional. The latter can for example be obtained by using well known immobilisation methods for the enzyme.
Before oxidation takes place the building blocks that form the starting composition in this reaction are functionalised with one or more phenolic residues containing an ortho methoxygroup.

Said functionalisation can be through natural occurrence of said phenolic groups in the building block or by synthetic functionalisation of said phenolic groups to the building block. It will be appreciated that the natural route is preferred.

In the oxidation reaction the phenolic groups are covalently linked. An example of a possible covalent link is shown in figure 1.

The oxidation may be carried out in any way known in the art . Both enzymatic oxidation and chemical oxidation routes may be used. Enzymatic oxidation is preferred. Suitable enzymes that can catalyse the formation of the covalent bond are peroxidase, laccase, polyphenol oxydases. Peroxidase is the preferred enzyme.

In case an enzymatic oxidising system is applied, the enzyme is preferably added in the form of a solution or a dispersion in an aqueous buffer system. The enzymes cited above are suitable enzymes. Some enzymes, such as peroxidases require the presence of a co-oxidant such as hydrogen peroxide for their activity. The co-oxidant is preferably added separately from the enzyme that requires it's presence. Alternatively a peroxide may be generated in situ by e.g. glucose oxidase and glucose.

The amount of enzyme added is expressed in terms of activity units. Preferably enzyme is present in excess.

If the oxidation is carried out enzymatically, the temperature during oxidation is preferably from 20 to 60 °C. Most preferred the temperature is around the temperature at which the enzyme shows maximum activity.

In case a chemical oxidant is applied, the oxidant is preferably added in the form of a diluted aqueous solution.

It will be appreciated that the exact reaction conditions in terms of temperature, ratio between the building blocks and type/amount of catalyst/enzyme, determine the final product composition in terms of polymer length and composition.

The invention is illustrated by the following examples.

Examples

Example 1

Triglyceride containing encapsulates have been produced with cross-linked pectin and cross-linked pectin linked via a Schiff base coupling to caseinate. Both encapsulates have been incubated under stomach conditions and did not show any lipid release, whereas incubations in the presence of pancreatic enzymes the mixed encapsulate (cross-linked protein + pectin) shows a release of the total lipid content within two hours. Under the same conditions the pectin encapsulate did not release any lipid within 90 minutes and only a limited amount after two hours.

Example 2

38 mg vanillin (ex Quest, food grade) was dissolved in 100ml water (stirring, approx.40 C).
1 gram sodium Caseinate (ex DMV, 95 % protein) was dissolved in above.
pH was adjusted to 8 and composition was stirred for 1 h at room temperature.

The product was stored overnight in freezer at -20 °C. Subsequently the composition was lyophilised (two days). The lyophilised product was dissolved in either 0.1M phosphate buffer pH7 or 0.1M Tris buffer pH 9 at a protein concentration of 10 mg/ml.

Crosslinking was effected with peroxidase from *Arthromyces Ramosus (ARP)* and H_2O_2. ARP was used in a concentration of 0.3 mg/ml batch. 1 microlitre was added for each ml protein solution. Hydrogenperoxide concentration was 1mM. Incubation was carried out at room temperature.

After 10 min, 30 min, 1 h and 18h incubation samples were taken and heated for 10 minutes to inactivate possible proteolytic enzymes. 50 microliter sample was mixed with 250 microliter SDS sample buffer, heated at 95°C for 15 minutes and submitted to SDS-PAGE (Exelgel 8/18).

The SDS gel electrophoresis results confirmed that after 10 minutes crosslinking had occurred in all cases. Further incubation did not give more crosslinking.

Crosslinking at pH 7 was more efficient than at pH 9. This could be explained by the fact that pH 7 is closer to the pH optimum of ARP.

After overnight incubation the sample was more viscous (visual inspection) than the sample without added enzyme also indicating formation of high molecular mass material.

Example 3

Covalent coupling of vanillin to ovalbumin by freeze drying

10 mg vanillin was dissolved in water by heating to approximately 40°C.

1 gram of ovalbumin (same as above) was added, pH adjusted to 8, stirring for 1h at room temperature. Freeze drying over weekend.

Crosslinking of ovalbumin-Vanillin / Analysis by electrophoresis

The obtained ovalbumin functionalised with vanillin was dissolved in water at a concentration of 50 mg/ml and the pH was adjusted to pH 8.0. To 1 ml sample was added: 1 µL 1 M hydrogenperoxide (final concentration 10 mM) and 1µL of a 0.3mg ARP/ml stocksolution . The reaction was allowed to proceed at room temperature overnight. Samples with and without crosslinking were diluted in SDS sample buffer to a final concentration of 0.5 mg ovalbumin-V/ml and analysed by SDS-PAGE (ExcelGel 8/18, Pharmacia).

Example 4

Chitosan (10 gram, Sample 973520, ex Primex, obtained via Snick Ingredients, Brugge) was dissolved in 1 liter aqua dest., containing 1% acetic acid.
After the chitosan had dissolved the pH was (slowly) raised by addition of 1N NaOH to 5.95.
Next the chitosan solution was divided in five 200ml portions and 4, 10, 20, 40 or 200 mg of vanillin (lignin vanillin ex Quest) was added to create building blocks with a varying degree of substitution with vanillin via Schiff base coupling. Vanillin was dissolved by warming to 40°C. Subsequently the solutions were freeze dried.

Of the freeze dried material 2 ml solutions containing 2% (w/v) modified chitosan in water were prepared. The pH of the solutions was close to pH=6.

Hydrogen peroxide was added to obtain a final concentration of 2mM and finally 5 microliter of a stock solution of soybean peroxidase (100mg QA00105076 ex Quest per ml water) was added.

Result: Extent of gelation was clearly dependent on degree of substitution.

Sample	vanillin:chitosan ratio (w:w)	Gelation
A	1:10	* *
B	1:50	+++
C	1:100	++
D	1:200	+
E	1:500	-

*= not all vanillin could be dissolved.

+++ = solid

++ = very high viscosity

+ = viscous solution

- = no visible increase in viscosity but rheological change of sample noticeable.

Example 5

<u>Oxidation of hydroyl-groups in starch to aldehyde groups.</u>

The method was essentially as described in US6346401.

Materials:

12 gram native wheat starch (Excelsior ex Avebe).

500 ml phosphate (0.75 g/l KH_2PO_4; pH 6)

1 ml Laccase (PPL ex NOVO, 6.6 mg/ml)

25 mg TEMPO (2,2,6,6-tetra-methyl-1-piperidinyloxy free radical, ex Fluka cat #87903)

These were mixed for 3h at 56°C. Starch granules were collected by centrifugation and washed with water. A qualitative test for aldehyde groups using dinitrophenylhydrazine showed that aldehyde-groups were successfully introduced in the starch granules.

Introduction of amino groups

After washing 12 gram of the above-prepared aldehyde starch was suspended in 100ml water.

500 microliter 1,2-diaminoethaan was added and pH adjusted to 6.

Schiffs base formation was accomplished by removal of water by means of freeze drying.

Excess 1,2-diaminoethaan was removed by washing with water and in between centrifugation. Washing was repeated until supernatant was negative in a qualitative amino test using trinitrobenzenesulfonic acid (TNBS).

Coupling of Vanillin

Approximately 12 grams NH2-starch was suspended in 100ml water, heated to 40°C, pH adjusted to 6 and approximately 100mg of Vanillin was added.

Schiffs base formation was accomplished by removal of water by means of freeze drying.

Excess vanillin was removed from the starch granules by washing with ethanol. Supernatant was checked for the presence of free vanillin by means of HPLC analysis and washing was proceeded until no free vanillin could be detected.

Amylolysis test

Samples of Starch-V (0.5 ml of 1% suspensions in 0.02M phosphate buffer, 0.067M NaCl, pH 6.7) were heated for 10 minutes in an Eppendorf heater at 1400rpm at the following

temperatures: RT, 40, 50, 60, 70, 80, 90°C, followed by cooling in ice-water.

Sensitivity to amylolytic breakdown was measured by adding 100 µl amylase solution (10 mg/ml porcine pancreas amylase type 6B ex Sigma) to 500 µl samples. After 4h incubation at Room temperature samples were centrifuged and to 100 µl supernatant 100 µL dinitrosalicylic acid (DNSA) solution was added. After heating the mixtures for 5 minutes at 100°C the optical density at 540nm was measured.

Result

The relative rate of hydrolysis of starch was higher for native wheat starch than for the crosslinked starch-V conjugate according to the invention.

Claims

1. Polymer composition comprising at least 2 building blocks covalently linked via phenolic residues containing a methoxygroup positioned ortho with respect to the hydroxyl group, wherein the building blocks are selected from protein (P), glycerides (G) and polysaccharides (S) and wherein the covalent linkage via phenolic residues is between P-P, S-S, P-S, P-G, G-G, G-S or combinations thereof, characterised in that at least one of the phenolic residues is covalently bonded to a building block via a Schiff's base or the reduced form thereof.

2. Polymer composition according to claim 1 wherein the building blocks are proteins or polysaccharides or a combination thereof.

3. Polymer composition according to claim 2 wherein either all building blocks are saccharides or all building blocks are proteins.

4. Polymer composition according to claim 1 wherein the phenolic residue is selected from the group of vanillic acid, ferulic acid, coniferol.

5. Food product comprising a polymer composition according to any of claims 1-4.

6. Oil and water containing emulsion comprising a polymer according to any of claims 1-4.

7. Oil and water emulsion comprising from 20 to 80 wt% fat and from 0.1 to 10 wt% of a polymer according to any of claims 1-4.

8. Oil and water emulsion according to claim 6 or 7 wherein the polymer comprises covalently linked glyceride and polysaccharide.

9. Use of the polymer according to any of claims 1-4 for encapsulation of compounds such as flavour compositions.

10. Method for the preparation of a polymer according to any of claims 1-4, wherein a composition comprising the building blocks is treated with an oxidative enzyme.

11. Use of a polymer according to any of claims 1-4 for targeted delivery of a functional molecule.

T7094 (V) cpl

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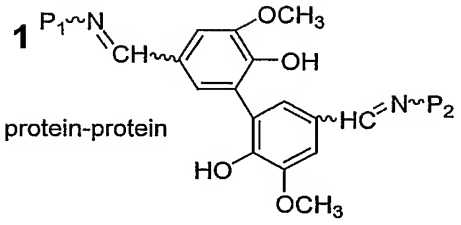

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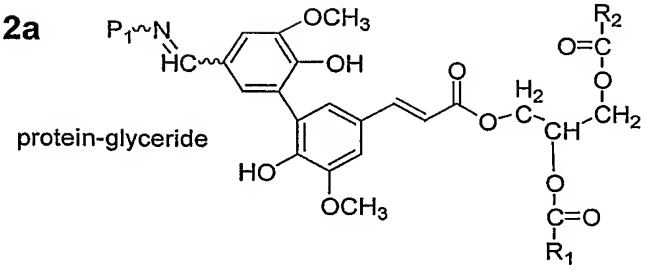

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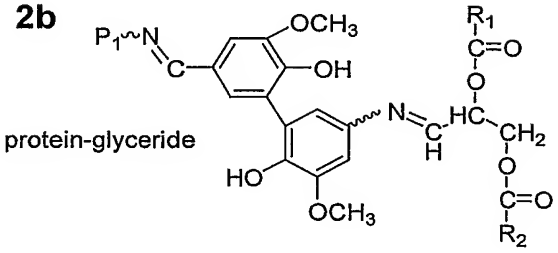

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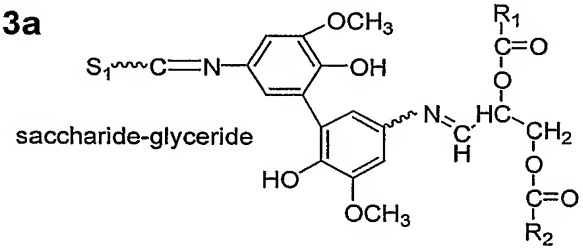

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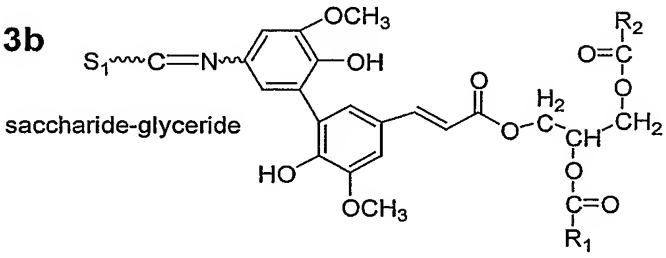


P= protein
S= polysaccharide
R= alkyl

3c

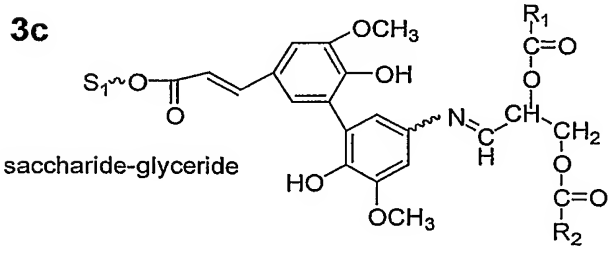
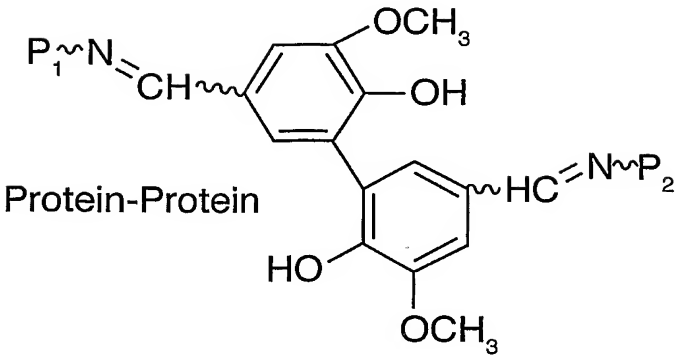


Fig.1.



P=Protien
S=Polysaccharide
R=Alkyl

Fig.2a.

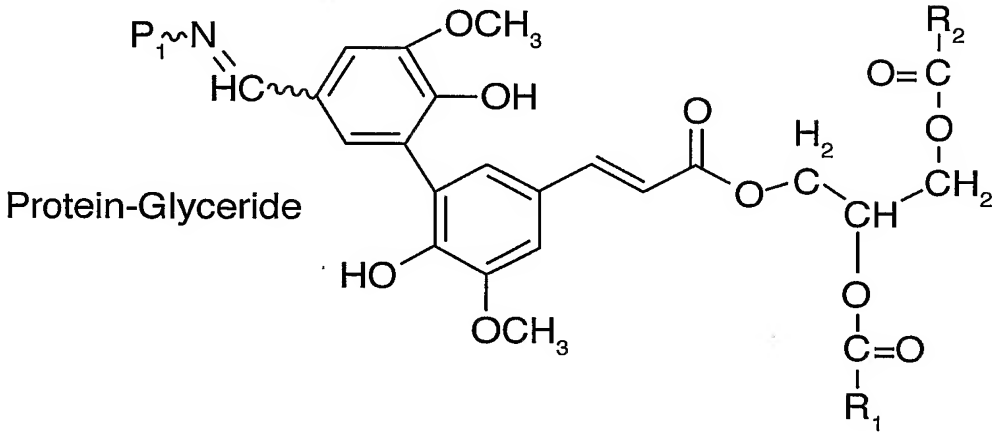


Fig.2b.

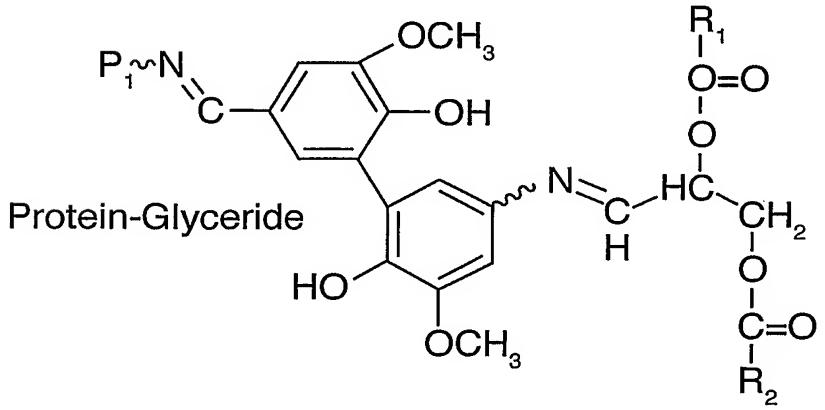
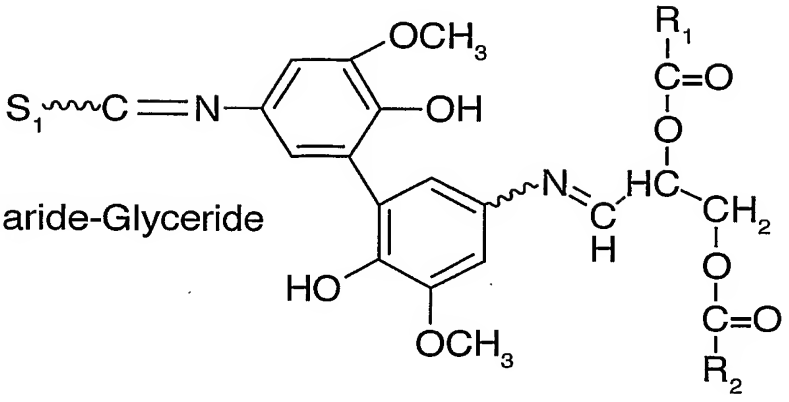


Fig.3a.



P=Protien
S=Polysaccharide
R=Alkyl

Fig.3b.

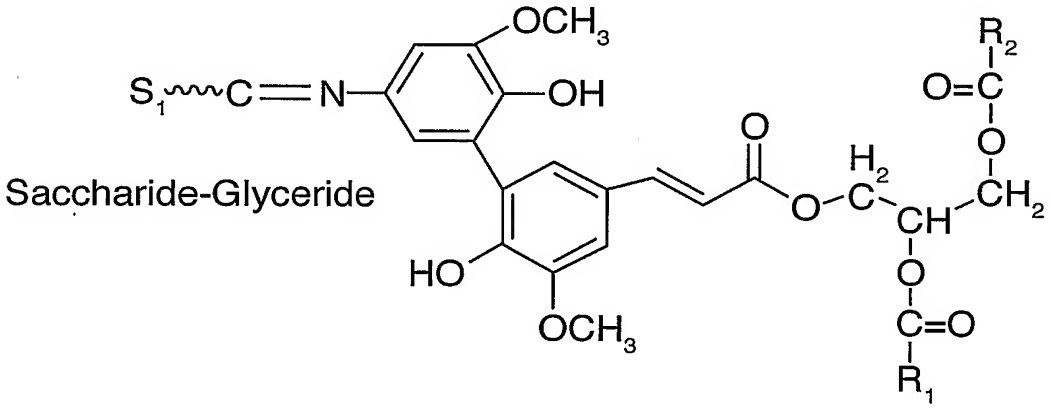
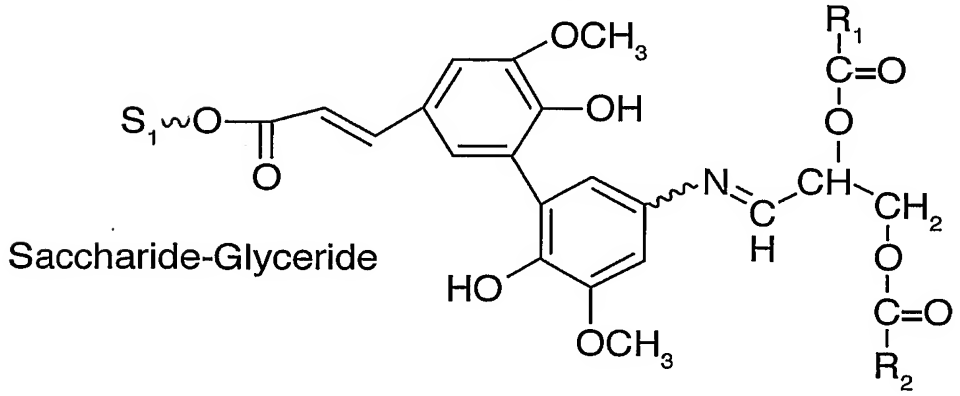


Fig.3c.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/002148

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/08 C08B37/06 C08H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C08B C08H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 374 441 A (SUZANNE M. GIBSON ET AL.) 20 December 1994 (1994-12-20) column 3, line 10 - line 66	1-5
Y	EP 1 169 922 A (ATO-DLO) 9 January 2002 (2002-01-09) cited in the application page 4, line 58; example 2	1-11
Y	US 4 925 677 A (JAN FEIJEN) 15 May 1990 (1990-05-15) column 4, line 45 - line 54	1-11
A	US 5 786 470 A (RODERICK GREENSCHIELDS ET AL.) 28 July 1998 (1998-07-28) cited in the application	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 2004	29/06/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lensen, H

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

[redacted]

International Application No
PCT/EP2004/002148

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FR 2 817 870 A (SKW NATURE PRODUCTS HOLDING FRANCE) 14 June 2002 (2002-06-14) cited in the application page 9	1-11
A	WO 00/11038 A (KUMAR GUNEET ET AL.) 2 March 2000 (2000-03-02)	
Y	US 6 303 585 B1 (ROBERT C. SPIRO ET AL.) 16 October 2001 (2001-10-16) column 2, line 43 - line 57	1-11
Y	GB 2 272 447 A (SANDOZ LTD) 18 May 1994 (1994-05-18) page 3, paragraph 5 page 6, paragraph 5 page 9, last paragraph	1-11

INTERNATIONAL SEARCH REPORT

[redacted]nformation on patent family members

International Application No
P[redacted]/EP2004/002148

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5374441 A	20-12-1994	NONE	
EP 1169922 A	09-01-2002	EP 1169922 A1	09-01-2002
US 4925677 A	15-05-1990	AT 87470 T	15-04-1993
		AU 649533 B2	26-05-1994
		AU 1616392 A	09-07-1992
		AU 622699 B2	16-04-1992
		AU 4012689 A	08-03-1990
		CA 1338958 C	04-03-1997
		DE 68905722 D1	06-05-1993
		DE 68905722 T2	08-07-1993
		EP 0357401 A2	07-03-1990
		ES 2054010 T3	01-08-1994
		IE 62977 B1	08-03-1995
		JP 2188534 A	24-07-1990
		JP 2960079 B2	06-10-1999
		KR 166057 B1	15-01-1999
		US 5041292 A	20-08-1991
US 5786470 A	28-07-1998	GB 2261671 A ,B	26-05-1993
		US 6033712 A	07-03-2000
		US 6174549 B1	16-01-2001
		AT 189820 T	15-03-2000
		AU 2933592 A	15-06-1993
		AU 2948892 A	15-06-1993
		BG 63014 B1	31-01-2001
		BG 98769 A	30-06-1995
		CA 2123602 A1	27-05-1993
		CA 2123613 A1	27-05-1993
		DE 69230690 D1	23-03-2000
		DE 69230690 T2	08-06-2000
		DE 69231698 D1	29-03-2001
		DE 69231698 T2	27-09-2001
		DE 69232731 D1	12-09-2002
		DE 69232731 T2	03-04-2003
		DK 646135 T3	25-06-2001
		DK 612326 T3	05-06-2000
		EE 3004 B1	16-06-1997
		EP 0646135 A1	05-04-1995
		EP 0612326 A1	31-08-1994
		EP 0990443 A2	05-04-2000
		ES 2156116 T3	16-06-2001
		ES 2141729 T3	01-04-2000
		ES 2181361 T3	16-02-2003
		FI 942245 A	13-05-1994
		WO 9310157 A1	27-05-1993
		WO 9310158 A1	27-05-1993
		GR 3033214 T3	31-08-2000
		HU 67479 A2	28-04-1995
		JP 7501216 T	09-02-1995
		JP 7502057 T	02-03-1995
		NO 941813 A	13-05-1994
		RO 113041 B1	30-03-1998
		US 5530112 A	25-06-1996
		US 5633032 A	27-05-1997
		US 5876779 A	02-03-1999
		ZA 9208825 A	21-06-1993

INTERNATIONAL SEARCH REPORT

[redacted]formation on patent family members

International Application No
PCT/EP2004/002148

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2817870 A	14-06-2002	FR 2817870 A1	14-06-2002
WO 0011038 A	02-03-2000	AU 5781499 A	14-03-2000
		CA 2341470 A1	02-03-2000
		EP 1137673 A1	04-10-2001
		JP 2002523528 T	30-07-2002
		WO 0011038 A1	02-03-2000
US 6303585 B1	16-10-2001	US 2004077592 A1	22-04-2004
		US 2003012765 A1	16-01-2003
		AU 752800 B2	03-10-2002
		AU 8290998 A	25-01-1999
		CA 2294756 A1	14-01-1999
		CN 1268057 T	27-09-2000
		EP 1011690 A1	28-06-2000
		JP 2002509538 T	26-03-2002
		NZ 502134 A	28-03-2002
		WO 9901143 A1	14-01-1999
GB 2272447 A	18-05-1994	NONE	

DERWENT-ACC-NO: 2004-737220

DERWENT-WEEK: 200472

TITLE: Polymer composition useful in food products comprises at least two building blocks covalently linked via phenolic residues containing an ortho methoxy group where a phenolic residues is covalently bonded to building block via Schiff's base

INVENTOR: BRUGGEMAN Y E; RAVESTEIN P ; VAN DER HIJDEN H T W M

PATENT-ASSIGNEE: HINDUSTAN LEVER LTD[UNIL] , UNILEVER NV[UNIL] , UNILEVER PLC [UNIL]

PRIORITY-DATA: 2003EP-075817 (March 21, 2003)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE
WO 2004083256 A1	September 30, 2004	EN

DESIGNATED-STATES: AE AG AL AM AT AU AZ BA BB BG BR BW BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE EG ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NI NO NZ OM PG PH PL P T RO RU SC SD SE SG SK SL SY TJ TM TN TR TT TZ UA UG US UZ VC VN YU ZA ZM ZW AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IT KE LS LU MC MW MZ NL OA PL PT RO SD SE SI SK SL SZ TR TZ UG ZM ZW

APPLICATION-DATA:

PUB-NO	APPL-DESCRIPTOR	APPL-NO	APPL-DATE
WO2004083256A1	N/A	2004WO-EP002148	March 4, 2004

INT-CL-CURRENT:

TYPE	IPC DATE
CIPS	C08B37/00 20060101
CIPS	C08B37/08 20060101
CIPS	C08H1/00 20060101

ABSTRACTED-PUB-NO: WO 2004083256 A1

BASIC-ABSTRACT:

NOVELTY - Polymer composition comprises at least 2 building blocks covalently linked via phenolic

residues containing methoxy group positioned ortho to the hydroxyl group. Building blocks are selected from protein (P), glycerides (G) and polysaccharides (S). Covalent linkage via phenolic residues is between P-P, S-S, P-S, P-G, G-G and/or G-S.

DESCRIPTION - A polymer composition comprises at least 2 building blocks covalently linked via phenolic residues containing methoxy group positioned ortho to the hydroxyl group. The building blocks are selected from protein (P), glycerides (G) and polysaccharides (S). The covalent linkage via phenolic residues is between P-P, S-S, P-S, P-G, G-G and/or G-S. At least one of the phenolic residues is covalently bonded to a building block via Schiff's base or its reduced form.

INDEPENDENT CLAIMS are included for the following:

(1) oil and water emulsion comprising (wt.%) fat (20 - 80) and the polymer (0.1 - 10);

(2) preparation of the polymer involving treating a composition comprising the building blocks with an oxidative enzyme.

None given.

USE - In food products, oil and water containing emulsions; for encapsulation of compounds such as flavor compositions; and for targeted delivery of a functional molecule (all claimed). Also useful in pharmaceutical products; and as texturising agents imparting viscosity and structure to a composition.

ADVANTAGE - The composition has superior emulsifying properties. The polymers are stable in the gastrointestinal tract and are only broken down with

release of the encapsulate, once specific enzymes are present.

EQUIVALENT-ABSTRACTS:

ORGANIC CHEMISTRY

Preferred Components: The building blocks are proteins and/or polysaccharides. Either all building blocks are saccharides or all building blocks are proteins. The phenolic residue is selected from vanillic acid, ferulic acid and coniferol.

POLYMERS

Preferred Components: The polymer comprises covalently linked glyceride and polysaccharide.

Chitosan (10 g) was dissolved in 1 liter aqua dest containing acetic acid (1%). After the chitosan had dissolved, the pH was slowly raised by addition of 1N NaOH to 5.95. Next the chitosan solution was divided in five 200 ml portions and 4,10, 20,40 or 200 mg of vanillin (lignin vanillin ex Quest) was added to create building blocks with a varying degree of substitution with vanillin via Schiff base coupling. Vanillin was dissolved by warming to 40 degrees C. Subsequently the solutions were freeze-dried. Of the freeze dried material solutions (2 ml) containing modified chitosan (2 w/v.%) in water were prepared. The pH of the solutions was close to 6. Hydrogen peroxide was added to obtain a final concentration of 2mM and finally a stock solution (5 microL) of soybean peroxidase (100 mg) was added. For the samples having a vanillin:chitosan weight ratio of 1:10, 1:50, 1:100, 1:200, and 1:500 respectively, the extent of gelation was not all vanillin could be dissolved, solid, very high viscosity, viscous solution and no visible increase

in viscosity but rheological change of sample noticeable.

TITLE-TERMS: POLYMER COMPOSITION USEFUL FOOD PRODUCT COMPRISE TWO BUILD BLOCK COVALENT LINK PHENOLIC RESIDUE CONTAIN ORTHO METHOXY GROUP BOND SCHIFF BASE

DERWENT-CLASS: A11 A97 B04 D13 D16

CPI-CODES: A03-A01; A12-W09; B04-B01B; B04-B01C; B04-C02; B04-N04; B12-M03; D03-H01D; D03-H01N; D05-A02A;

CHEMICAL-CODES: Chemical Indexing M1 *01* Fragmentation Code M417 M423 M710 M720 N103 N134 N411 Q211 Q233 Specific Compounds RA00I9 Registry Numbers 184613

Chemical Indexing M1 *02* Fragmentation Code G017 G019 G100 H4 H402 H442 H5 H542 H8 K0 L3 L355 L399 M1 M111 M210 M211 M272 M282 M320 M423 M510 M520 M532 M540 M710 M720 N103 N134 N411 Q211 Q233 Markush Compounds 014123701

Chemical Indexing M1 *03* Fragmentation Code G017 G019 G100 H4 H402 H442 H5 H542 H8 J0 J012 J2 J272 K0 L3 L355 L399 M1 M111 M210 M211 M212 M213 M214 M215 M216 M220 M221 M222 M223 M224 M225 M226 M231 M232 M233 M262 M272 M282 M312 M321 M332 M343 M349 M381 M391 M423 M510 M520 M532 M540 M710 M720 N103 N134 N411 Q211 Q233 Markush Compounds 014123702

Chemical Indexing M1 *04*
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Chemical Indexing M6 *05*
Fragmentation Code Q211 Q233 R022 R111 R120 R310

ENHANCED-POLYMER-INDEXING: Polymer Index [1.1]
2004 ; G3623*R P0599 D01; M9999 M2391; L9999 L2391; L9999 L2324; M9999 M2324;

Polymer Index [1.2]
2004 ; D01 D11 D10 D23 D22 D31 D42 D50 D76 D86 F08 F07 F24 F28 F26 F34 H0293 P0599 G3623 M2313 R03882 104328; M9999 M2391; L9999 L2391; L9999 L2324; M9999 M2324;

Polymer Index [1.3]
2004 ; ND04; Q9999 Q7590 Q7589; ND03; ND06; Q9999 Q7523; Q9999 Q8037 Q7987; Q9999 Q9347; B9999 B3372*R; B9999

B4579 B4568; Q9999
Q7250; B9999 B3554*R;

Polymer Index [1.4]
2004 ; C999 C044 C000;
C999 C271;

SECONDARY-ACC-NO:

CPI Secondary Accession Numbers: 2004-259171